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FORM A
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TO THE MINISTRY OF INDUSTRY COMMERCE AND HANDICRAFT
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Patent Application for Industrial Invention, filing of reserves,
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D. TITLE proposed class, (sec./cl./ucl.) group/subgroup

Use of inhibitors of the protease of the human immunodeficiency virus (HIV) in the therapy of Kaposi's sarcoma, of tumours and of angioproliferative, inflammatory and autoimmune diseases, associated and not associated with HIV infection

ADVANCED OPENING TO PUBLIC INSPECTION yes___ no____
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1) ENSOLI Barbara 3)
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F. PRIORITY
Country or Exhibition Type of Priority Appln. No. Appln. date Encl(yes/res)

1) NONE

2)

G. CENTRE FOR COLLECTING MICROORGANISMS'CULTURES, denomination

None

H. SPECIAL NOTES
None

ENCLOSED DOCUMENTS

					RESERVES DISSOLUTION
Specimen No.					date No. of ref.
Doc. 1) 2	prov.	no. sheets 44	abstract with main drawing, spec. and claims (compulsory 1 copy)		
Doc. 2) 2	prov.	no. sheets 11	(compulsory if cited in description., 1 copy)		
Doc. 3) 1	res.		power of attorney or reference attorney		
Doc. 4) 0	res.		designation of inventor		
Doc. 5) 0	res.		priority document with Italian translation		comparison single priority
Doc. 6) 0	res.		authorisation or assignment deed		
Doc. 7) 0	res.		complete name of the applicant		

8) PAYMENT RECEIPT OF LIT. 915.000.= compulsory
filled in on 18.04.2001

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follows yes/no no
We required certified copy of the present deed yes/no yes

PROVINCIAL OFFICE OF INDUSTRY COMMERCE HANDICRAFT OF ROME code 58
FILING CERTIFICATE Application no. RM2001A000210 Reg. A
The year 2001 the 18th day of the month of April

The above mentioned applicant(s) has(have) presented to me undersigned the present application consisting of no. 00 additional sheets for the grant of the above patent.

I. DIFFERENT NOTES OF THE RECORDING OFFICER
none

THE DEPOSITER
(signature)

THE RECORDING OFFICER
(signature)
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FORM A

ABSTRACT OF THE INVENTION TOGETHER WITH MAIN DRAWING, SPECIFICATIONS AND CLAIMS

Application No. RM2001A000210 Reg. A
Patent No.

Filing date 18/04/2001
Date of grant

Applicant (I)
Name
Residence

D. TITLE

Use of inhibitors of the protease of the human immunodeficiency virus (HIV) in the therapy of Kaposi's sarcoma, of tumours and of angioproliferative, inflammatory and autoimmune diseases, associated and not associated with HIV infection

L. ABSTRACT

The present invention refers to the use of inhibitors of the protease of the human immunodeficiency virus (HIV) (HIV-PI) as drugs or for the preparation of new drugs having anti-angiogenic, anti-oedemigenic, anti-inflammatory for the treatment of Kaposi's sarcoma, of tumours and of angioproliferative, inflammatory and autoimmune diseases, associated and not associated with HIV infection

M. DRAWING

Description

of the invention for industrial invention having for title:
Use of inhibitors of the protease of the human immunodeficiency virus (HIV) in the therapy of Kaposi's sarcoma, of tumours and of angioproliferative, inflammatory and autoimmune diseases, associated and not associated with HIV infection
in the name of ISTITUTO SUPERIORE DI SANITA'
having seat in 00161 ROMA – Viale Regina Elena 299
named inventor: Barbara ENSOLI
filed on under No.

* * * * *

Use of inhibitors of the protease of the human immunodeficiency virus (HIV) in the therapy of Kaposi's sarcoma, of tumours and of angioproliferative, inflammatory and autoimmune diseases, associated and not associated with HIV infection

Field of the invention

The present invention refers to the use of inhibitors of the protease of the human immunodeficiency virus (HIV) in the therapy of Kaposi's sarcoma, of tumours and of angioproliferative, inflammatory and autoimmune diseases, associated and not associated with HIV infection.

Prior art

The inhibitors of the protease of the HIV virus are compounds with a known activity and are described, for example, in Deeks et al. (Deeks et al., 1997). They are used in the therapy of HIV infection in subjects affected by the acquired immunodeficiency syndrome (AIDS) with the function of inhibiting the maturing of the virus and blocking its replication (Deeks et al., 1997). In this description the inhibitors of the protease of the HIV virus will also be indicated below with the abbreviation HIV-PI.

Kaposi's sarcoma (KS) is a tumour associated with infection by the human herpesvirus 8 (HHV8) and is particularly frequent in subjects infected with the HIV virus (AIDS, KS) (Ensoli and Stürzl, 1998; Ensoli et al., in press). KS is also observed in subject not infected with HIV, particularly in the Mediterranean area and in Italy (classic KS), in Africa (endemic KS) and in organ-transplanted subjects subjected to immunosuppressive therapy (iatrogenic KS) (Ensoli and Stürzl, 1998; Ensoli et al., in press). The deregulation of the immune system seems to be a necessary condition for the development of KS in subjects infected with the virus HHV8 (Ensoli and Stürzl, 1998; Ensoli et al., in press).

Various authors have described a reduced incidence of KS and of lymphomas (International Collaboration on HIV and Cancer, 2000) or regression (Lebbé et al., 1998; Cattelan et al., 1999) of KS in patients infected with HIV and treated with combinations of antiretroviral drugs containing at least one HIV-PI (Deeks et al., 1997). KS is a vascular tumour characterised by angiogenesis, vascular permeability and oedema, growth of cells of endothelial origin (KS cells) and by

infiltration of inflammatory cells, and it is particularly frequent and aggressive in homosexual and bisexual males infected with HIV and jointly infected with HHV-8 (Ensoli and Stürzl, 1998; Ensoli et al., in press). The formation of the lesions is mediated by cytokines with angiogenic, proliferative oedemigenic and inflammatory effects, produced by KS cells, by activated endothelial cells and by immune cells infiltrating the tissues (Ensoli et al., 1989; Ensoli et al., 1994a; Ensoli et al., 1994b; Fiorelli et al., 1995; Samaniego et al., 1995; Samaniego et al., 1997; Samaniego et al., 1998; Barillari et al., 1999a). Among the angiogenic factors, the basic fibroblast growth factor (bFGF) is expressed at high levels in the lesions and is the most important autocrine and paracrine factor for the growth of KS and for angiogenesis (Ensoli et al., 1989; Ensoli et al., 1994a; Ensoli et al., 1994b; Samaniego et al., 1995; Fiorelli et al., 1995; Samaniego et al., 1997; Samaniego et al., 1998; Barillari et al., 1999a). In fact, antibodies or antisense oligomers directed against the bFGF block both the angiogenesis and the formation of KS-like lesions promoted by the inoculation of primary KS cells in nude mice but also the growth of KS cells in vitro (Ensoli et al., 1989; Ensoli et al., 1994b; Barillari et al., 1999b). Vice versa, the inoculation of bFGF in nude mice promotes the development of KS-like angioproliferous lesions (Ensoli et al., 1994a; Samaniego et al., 1998; Barillari et al., 1999a), the frequency and aggressiveness of which are increased by the protein Tat of HIV-1, which is able to mimic the action of proteins of the extracellular matrix. In particular, to act on the KS, Tat requires the presence of bFGF or of inflammatory cytokines which induce the production of bFGF which in turn promotes the expression of the receptors for Tat (Ensoli et al., 1990; Barillari et al., 1992; Barillari et al., 1993; Ensoli et al., 1994a; Albini et al., 1995; Fiorelli et al., 1995; Fiorelli et al., 1998; Fiorelli et al., 1999; Barillari et al., 1999a and 1999b). Another inducer of growth, angiogenesis and vascular permeability present in KS is the Vascular endothelial growth factor (VEGF), which cooperates with bFGF in the angiogenesis and oedema of KS (Samaniego et al., 1998). Other factors present in KS and which cooperate in its formation are interleukyne (IL)-1, IL-6. the tumour necrosis factor (TNF)α, interferon (IFN)γ, the granulocyte-monocyte colony-stimulating factor (GMCSF), the platelet-derived growth factor (PDGF), oncostatin-M and the β-chemokynes (RANTES, MIP-1α,

MIP-1β, and others) (Ensoli and Stürzl, 1998; Ensoli et al., in press). In particular, the inflammatory cytokines such as IL-1, IL-6, TNFα and IFNγ induce KS cells and endothelial cells to produce bFGF and VEGF, induce endothelial cells to acquire the phenotype of KS cells, they are angiogenic in vivo and induce the increase of KS lesions in mice (Samaniego et al., 1995; Fiorelli et al., 1995; Fiorelli et al., 1998; Barillari et al., 1999a). As well as promoting the growth of KS, bFGF, like VEGF, is able to activate all the processes that are required for inducing angiogenesis. Angiogenesis in turn is fundamental for the growth and metastasis of tumours and for non neoblastic angioproliferative diseases and is often an important component in chronic inflammatory diseases (Carmeliet and Jain, 2000). Angiogenesis requires 3 distinct processes: the degradation of the vascular basal membrane by endothelial proteases, the directional migration of cells in the perivascular space (invasion and migration of endothelial cells) and the proliferation of endothelial cells (Carmeliet and Jain, 2000). In particular, the degradation of the vascular basal membrane is mediated by the metalloproteases of the matrix (MMP). The MMP themselves are necessary for tumoural and metastatic growth and for the infiltration of inflammatory cells in the tissues (Stetler-Stevenson, 1999). Among these, MMP-2 is essential for angiogenesis, it is induced by bFGF and is strongly expressed in primary lesions of KS and in other neoplasias (Ensoli et al., 1994a; Barillari et al., 1999b; Stetler-Stevenson, 1999; Toschi et al., submitted). The inhibition of the migration, invasion or proliferation of endothelial cells, or of the activity of MMP-2 is able to block angiogenesis and constitutes the reasoning for the anti-angiogenic and anti-tumoural therapies currently in use (Stetler-Stevenson, 1999; Koivunen et al., 1999; Carmeliet and Jain, 2000).

The lower incidence and the regression of KS observed in individuals infected with HIV and treated with HIV-PI (Lebbé et al., 1998; Cattelan et al., 1999; International Collaboration on HIV and Cancer, 2000) has been related to this drug's capacity to inhibit the replication of HIV and, consequently, the production and release of the protein Tat of HIV-1, a powerful KS progression factor (Ensoli et al., 1990; Ensoli et al., 1994a; Barillari et al., 1999a; Barillari et al., 1999b). Moreover, by reconstituting the number and the function of specific cytotoxic T lymphocytes and

the natural killer activity, the treatment with HIV-PI increases a protective immune response against HHV-8 (Ensoli et al., in press), the virus considered to be the cause of KS (Ensoli and Stürzl, 1998; Ensoli et al., in press). In fact, in subjects treated with HIV-PI a reduction is observed in the viral charge both of HIV (Deeks et al., 1997) and of HHV-8 and the reappearance of the immunological responses against HHV-8 (Blum et al., 1997; Rizzieri et al., 1997; Lebbé et al., 1998; Osman et al., 1999; Sirianni et al., 1999; Sirianni et al., 2000; Wang et al., 2000).

However, we hypothesised that the lower incidence and the regression of KS observed in individuals treated with HIV-PI could be due to a direct anti-angiogenic, anti-tumoural, anti-oedemigenic and/or anti-inflammatory effect of these drugs. It must be remarked that these effects of HIV-PI could not be anticipated on the basis of the existing studies. In fact, all the studies agree in attributing the lower incidence or regression of KS in subjects treated with HIV-PI to the inhibition of the HIV infection with consequent reduction of the expression of the protein Tat, to the reconstruction of the immune system, or to the disappearance of the human herpesvirus 8 from the blood or from the lesions as a result of the reconstitution of humoural immune or effective cellulo-mediated responses against the virus (Blum et al., 1997; Rizzieri et al., 1997; Lebbé et al., 1998; De Milito et al., 1999; Cattelan et al., 1999; Osman et al., 1999; Sirianni et al., 1999; Sirianni et al., 2000; Wang et al., 2000). On the contrary, the effects of HIV-PI that we hypothesised have never been described or studied before.

Summary of the invention

It is an object of the present invention the use of the inhibitors of the protease of the HIV virus (HIV-PI) to produce drugs with an anti-angiogenic action for the treatment of tumours and of non neoblastic angioproliferous diseases in subjects not infected with the HIV virus.

Another object of the invention is the use of the inhibitors of the protease of the HIV virus (HIV-PI) to produce drugs with a direct anti-tumoural activity in subjects not infected with the HIV virus.

Another object of the invention is the use of the inhibitors of the protease of the HIV virus (HIV-PI) to produce drugs with an anti-oedemigenic, anti-inflammatory

action and for the therapy of inflammatory and autoimmune diseases in subjects not infected with the HIV virus.

Another object of the invention is the use of the inhibitors of the protease of the HIV virus (HIV-PI) to produce drugs for the treatment of Kaposi's sarcoma in subjects not infected with the HIV virus.

Another object of the invention is the use of the inhibitors of the protease of the HIV virus (HIV-PI) to produce drugs with an anti-angiogenic, anti-tumoural, anti-oedemigenic and/or anti-inflammatory action, for the treatment of Kaposi's sarcoma, of tumours and of non neoblastic angioproliferous, inflammatory and autoimmune diseases in subjects infected with the HIV virus.

A further object of the invention is the use for the above purposes of the compounds known as Crixivan® (indinavir) marketed by Merck, Sharp and Dohme; Invirase® or Fortovase® (saquinavir), marketed by Roche; Norvir® (ritonavir), marketed by Abbott Laboratories; Viracept® (nelfinavir), marketed by Roche; Agenerase® (amprenavir), marketed by Glaxo Wellcome; Kaletra® (lopinavir and ritonavir), marketed by Abbott Laboratories.

Another object of the invention is the use of the inhibitors of the protease of the HIV virus (HIV-PI) and of the compounds listed above for the above indications in combination with one another and/or in association with anti-inflammatory, anti-angiogenic or anti-tumoural drugs.

A further object of the invention is the use of similar substances or derivatives of the inhibitors of the protease of HIV, HIV-PI, and of the compounds listed above with anti-angiogenic, anti-tumoural, anti-oedemigenic and anti-inflammatory activity alone or combined with one another and/or in association with anti-inflammatory, anti-angiogenic or anti-tumoural drugs.

Further objects of the invention will be evident from the following detailed description of the invention.

Brief description of the Figures

Fig. 1 Indinavir and saquinavir inhibit the formation of angioproliferative lesions induced by bFGF in the nude mouse.

Fig. 2 Indinavir and saquinavir do not interfere with the proliferation of endothelial cells in response to bFGF.

Fig. 3 Indinavir and saquinavir inhibit the migration of endothelial cells in response to bFGF.

Fig. 4 Indinavir and saquinavir inhibit the invasion of endothelial cells in response to bFGF.

Fig. 5 Indinavir blocks the activation of MMP-2 in endothelial cells.

Fig. 6 Saquinavir blocks the activation of MMP-2 in endothelial cells.

Fig. 7 Indinavir and saquinavir promote the regression of KS-like lesions induced by the inoculation of KS cells in the nude mouse.

Fig. 8 Indinavir and saquinavir block the vascular permeability and the oedema promoted by the inoculation of KS cells in the nude mouse.

Fig. 9 Indinavir and saquinavir inhibit the invasive capacity of KS cells.

Fig. 10 Indinavir and saquinavir block the production of inflammatory cytokines such as IL-6 by KS cells.

Detailed description of the invention

The treatment of AIDS patients with combinations of antiretroviral drugs containing an inhibitor of the protease of the HIV virus (HIV-PI) (for example indinavir or saquinavir) has shown that it decreases the incidence of KS and of lymphomas and induces the regression of KS in the subjects treated (Lebbé et al., 1998; Cattelan et al., 1999; International Collaboration on HIV and Cancer, 2000). These effects have been attributed by others to the blocking, by HIV-PI, of the replication of the HIV virus, to the blocking of the replication of the HHV8 virus and/or to the reconstitution of effective immune responses against HHV-8 and HIV (Blum et al., 1997; Rizzieri et al., 1997; Lebbé et al., 1998; De Milito et al., 1999; Cattelan et al., 1999; Osman et al., 1999; Sirianni et al., 1999; Sirianni et al., 2000; Wang et al., 2000). Besides, our past and recent studies indicate that cytokines, growth and angiogenic factors [particularly the basic fibroblast growth factor (bFGF)] produced by KS cells, endothelial cells and cells of the immune system mediate the formation of KS lesions (Ensoli et al., 1989; Ensoli et al., 1994a; Fiorelli et al., 1995; Samaniego et al., 1995; Samaniego et al., 1997; Ensoli and Stürzl, 1998; Fiorelli et al., 1998; Samaniego et al., 1998; Barillari et al., 1999a; Fiorelli et al., 1999; Ensoli et al., in press), So, contrary to the opinion commonly held in the scientific world, we hypothesised that in the combination of drugs administered to

subjects with AIDS and KS, the effect of regression on KS was due to a direct activity of HIV-PI on angiogenesis, on the growth of the tumour, on oedema and on inflammation. Using models in vivo and in vitro, we demonstrated that indinavir and saquinavir block the development of KS-like angioproliferative lesions induced by the inoculation of bFGF or of primary human KS cells in nude mice. This effect is due to the block of the migration and invasion of endothelial and tumoural cells and is associated with the inhibition of the activation of an enzyme called metalloprotease-2 (MMP-2) by indinavir or saquinavir used in the same concentrations present in human plasma. The enzymes of the metalloprotease class are essential for cell motility (migration and invasion) and, therefore, for the angiogenesis, growth and invasion of tumours (Carmeliet and Jain, 2000). Moreover, HIV-PI block the vascular permeability and the oedema promoted by the KS cells in the nude mouse and the production of cytokine by KS cells. These cytokines not only contribute to the promotion of KS lesions but they also have inflammatory activity (Ensoli et al., 1989; Barillari et al., 1992; Samaniego et al., 1995; Fiorelli et al., 1995; Samaniego et al., 1997; Sirianni et al., 1998; Samaniego et al., 1998; Fiorelli et al., 1998; Fiorelli et al., 1999; Barillari et al., 1999a) and some of them act by promoting the growth of the multicentric disease of Castleman and of lymphomas (Tosato et al., 1993; Peterson and Frizzera, 1993; Ramsay et al., 1994; Asou et al., 1998). These data, therefore, indicate that the effect of HIV-PI on KS and on lymphomas is due to a direct block on the MMP-2, on the migration and invasion of endothelial and tumoural cells, on angiogenesis, on oedema and on the production of cytokines, determining the inhibition of the formation of lesions and the lower incidence of tumours observed in the murine model and/or in subjects treated with HIV-PI.

It is important to stress that these therapeutic effects were obtained in the absence of HIV and HHV8, therefore excluding that the effects of HIV-PI on KS, angiogenesis, vascular permeability and oedema, inflammation and the production of cytokines could be mediated by the effects of HIV-PI on HIV and/or HHV-8.

The discovery that the inhibitors of HIV protease are powerful anti-angiogenic, anti-tumoural, anti-oedemigenic and anti-inflammatory drugs and that they block the activation of cellular proteases involved in these processes, opens up a

completely new field and therefore represents a new and different therapeutic indication of these drugs for their use against angiogenesis, non neoblastic angioproliferous pathologies, KS, tumours and inflammatory and autoimmune diseases, both in HIV-infected subjects and in non HIV-infected subjects.

All those compounds that present activity as inhibitors of the protease of the HIV virus (indicated for brevity's sake as HIV-PI) and similar to or derived from the same therefore fall within the field of the present invention. Purely as an example, those known as indinavir, saquinavir, ritonavir, nelfinavir, amprenavir, lopinavir are mentioned.

HIV-PI compounds may be used as follows in both HIV-infected and non HIV-infected subjects:

- for the therapy of Kaposi's sarcoma (KS)
- for the therapy of angiogenesis
- for the therapy of non neoblastic angioproliferous diseases (eye, kidney, vascular system, skin), such as, for example, diabetic retinopathy, retrodental fibroplasia, trachoma, vascular glaucoma, psoriasis, immune and non immune inflammation, atherosclerosis, keloids
- for the therapy of benign and malignant tumours of the soft tissues, the cartilages, the bones and the blood
- for blocking the activity of the bFGF with a therapeutic anti-angiogenic, anti-tumoural, anti-KS effect
- for blocking the activity of the VEGF with a therapeutic anti-angiogenic, anti-tumoural, anti-KS, anti-oedemigenic effect
- for blocking the activity of associated bFGF and VEGF with a therapeutic anti-angiogenic, anti-tumoural, anti-KS, anti-oedemigenic effect
- for blocking the activity of Tat alone or in the presence of bFGF with a therapeutic anti-angiogenic, anti-tumoural, anti-KS, anti-oedemigenic and anti-inflammatory effect
- for blocking the migration of endothelial cells with a therapeutic anti-angiogenic, anti-KS and anti-tumoural effect
- for blocking the migration of tumoural cells with a therapeutic anti-KS and anti-tumoural effect

- for blocking the invasion of endothelial cells with a therapeutic anti-angiogenic, anti-KS and anti-tumoural effect
- for blocking the invasion of tumoural cells with a therapeutic anti-KS and anti-tumoural effect
- for blocking MMP-2 and the other proteases involved in angiogenesis (Carmeliet, Nature 2000)
- for blocking MMP-2 and other proteases involved in the growth and metastasis of tumours
- for blocking vascular permeability and oedema associated with angiogenesis
- for blocking vascular permeability and oedema associated with tumours
- for blocking vascular permeability and oedema associated with KS
- for blocking vascular permeability and oedema associated with inflammation
- for blocking the production of inflammatory cytokines with a therapeutic anti-inflammatory effect
- for blocking the production of cytokines with a therapeutic anti-oedemigenic effect
- for blocking the production of cytokines with a therapeutic anti- angiogenic effect
- for blocking the production of cytokines with a therapeutic anti-KS effect
- for blocking the production of cytokines with a therapeutic anti-tumoural effect
- for the therapy of autoimmune diseases in general, in particular systemic lupus erythematosus, scleroderma, rheumatoid arthritis, psoriasis, thyroiditis, ulcerous rectocolitis and Crohn's disease, Goodpasture's syndrome, systemic vasculitis, Sjögren's syndrome, primitive biliary cirrhosis
- for the therapy of inflammatory diseases, in particular of chronic inflammation associated with allergies and with viral infective, bacterial or parasitic agents, including the multicentric disease of Castleman.

For the above-mentioned uses all those compounds which manifest activity that inhibits the protease of the HIV virus are generally indicated, while particularly indicated are the compounds called indinavir, saquinavir, ritonavir, nelfinavir, amprenavir, lopinavir and those similar to or derived from them, alone or in a combination with one another and/or in combination with other drugs.

The HIV-PI according to the invention may be given by oral, intravenous, intramuscular, subcutaneous, intradermal, intraperitoneal, intrathecal, intrapleural, intrauterine, intravaginal, topic intrarectal, transmucosal, intralesional or percutaneous administration, for all the indications listed above. The doses and the means of administration depend on the type of affection to be treated. In particular, doses are considered that are lower , equal to or higher than those commonly used for the treatment of HIV-infected patients. For example these doses are, for indinavir: 1200 mg/day, 2400 mg/day and 4800 mg/day; and for saquinavir: 1800 mg/day, 3600 mg/day, 7200 mg/day.

The examples given below, which also refer to the figures and tables enclosed, may be used for verifying our hypothesis. We used indinavir and saquinavir, two HIV-PI associated with the regression of KS in treated patients (Lebbé et al., 1998; Cattelan et al., 1999), with a similar structure but with chemical substituents designed to optimise their action. The effects of both the HIV-PI were studied in models of angiogenesis promoted by bFGF in vivo and in vitro, on the formation of KS-like lesions and on vascular permeability induced in vivo by KS cells and on KS cells in vitro (Ensoli et al., 1989; Ensoli et al., 1994a and 1994b; Samaniego et al., 1995; Fiorelli et al., 1995; Samaniego et al., 1997; Samaniego et al., 1998; Barillari et al., 1999a; Sgadari et al., 2000).

The present invention will therefore be described in relation to the following examples which are to be considered illustrative and without limitation of the scope of the same, referring to the enclosed figures and tables.

Materials and methods/Detailed description of the figures

Fig. 1. Indinavir and saquinavir block the formation of KS-like angiogenic lesions induced by bFGF in nude mice.

(A) panel a, mice injected on the two sides with a buffer (PBS-0.1% BSA) in matrigel and treated with saline solution; panel b, mice injected on the two sides with bFGF(1 μg) in matrigel and treated with saline solution; panel c, mice injected with bFGF(1 μg) in matrigel and treated with indinavir (1.4 mg/day); panel d, mice injected with bFGF(1 μg) in matrigel and treated with saquinavir (1 mg/day). (B) panels a, b, 100X and 400X enlargements of the sites of inoculation stained with haematoxylin/eosin of the mice injected with a buffer and treated with saline

solution, respectively; panels c and d, 100X and 400X enlargements of the sites of inoculation of the mice injected with bFGF and treated with saline solution, respectively; panels e and f, 100X and 400X enlargements of the sites of inoculation of the mice injected with bFGF and treated with indinavir respectively; panels g and h, 100X and 400X enlargements of the sites of inoculation of the mice injected with bFGF and treated with saquinavir, respectively. The experiments were carried out (26) as described in the key to Table 1.

Fig. 2. Indinavir and saquinavir have no effect on the basal or bFGF-induced proliferation of primary endothelial cells.

The figure shows the results of the proliferation trial expressed as the number of cells counted after 5 days of incubation with bFGF (black bars) or buffer (white bars) in the presence or absence of 0.1, 1 or 10 μM of indinavir or saquinavir. Human endothelial cells from the umbilical vein (HUVEC, Bio-Whittaker, Verviers, Belgium) were plated in triplicate ($1.5x10^4$ cells/well) in plates of 12 wells previously covered with gelatine. The next day the cells were incubated for 4 hours in a medium without serum and cultivated in RPMI 1640 medium (Life Technologies, Eragny, France) with the addition of 10% of foetal bovine serum (FBS) together with bFGF (10 ng/ml) or its diluting buffer (PBS). The medium, the bFGF, the indinavir, the saquinavir or the buffer were replaced after 3 days. After 5 days' culture, the cells were counted after having been stained with trypan blue, as previously described (Ensoli et al., 1990; Ensoli et al., 1994b): For all the studies in vitro, the indinavir or the saquinavir in the pure powder formula (Merck-Sharpe & Dhome and Roche, respectively) were re-suspended in distilled water. The drugs were found to be free from endotoxins on LAL testing (Associated of Cape Code Inc., Falmouth, MA).

Fig. 3 and 4. Indinavir and saquinavir block the migration and the invasion of endothelial cells induced by bFGF. Figure 3 shows the results of the migration trial and figure 4 shows the results of the invasion trial of the endothelial cells expressed, respectively, with the number of cells/well that migrated (Fig. 3) or invaded (Fig. 4) in response to the bFGF (black bars) or to its diluting buffer (white bars) in the presence 0.1, 1 or 10 μM of indinavir or saquinavir or of the diluting buffer.

Both trials were performed by means of a Boyden chamber separated in two compartments by polycarbonate filters with pores with a diameter of 12 µm (Nucleoprobe, Cabin John, MD), covered with collagen IV (Collaborative Biomedical Products) for the migration trial, or with collagen IV and Matrigel together for the invasion trial, as described previously (Barillari et al., 1999b). The HUVEC were cultivated for 5-6 days in the presence of scalar concentrations of indinavir or saquinavir, or the diluting buffer (control). The cells were collected, re-suspended in a medium without serum containing 0.01% of BSA and placed in the top compartment of the Boyden chamber in duplicate ($2x10^5$ cells/well) in the presence of indinavir, saquinavir or of the diluting buffer. bFGF (50 ng/ml) was placed in the bottom compartment as a chemoattractant in a medium containing 0.01% BSA. After 5 hours (migration) or 6 hours (invasion) of incubation, the non migrated cells present on the top surface of the filters were mechanically removed, while the migrated cells on the bottom surface were fixed in methanol and stained with toluidine blue (Sigma Chemical Co., St. Louis, MO). The cells present in 5-10 microscopic fields of the filters, chosen at random, were counted as described previously (Barillari et al., 1999b).

Fig. 5 and 6. Indinavir and saquinavir block the conversion of latent MMP-2 into its active form. Figure 5 refers to indinavir and figure 6 to saquinavir.

(A) shows a trial of zymography carried out on concentrated supernatants coming from HUVEC stimulated with bFGF (black bars) or its re-suspension buffer (white bars), and cultivated for 24 hours in the presence of 0.1, 1 or 10 µM of indinavir or saquinavir, respectively. The arrows indicate the destained areas due to gelatinolithic activity corresponding to the latent forms (72 kD) or active forms (64 kD and 62 kD) of MMP-2. (B) and (C) indicate the densitometric quantification of the destained areas corresponding to the gelatinolithic activity of the latent forms 72 kD or active forms 64-62 kD of MMP-2 released by the cells. The results are expressed as the optical density of the destained bands.

The HUVEC were cultivated for 24 hours in RPMI 1640 with the addition of 10% FBS in the presence of scalar concentrations of indinavir, saquinavir or the diluting buffer, in the absence or presence of bFGF (100 ng/ml). The cells were then washed twice with a medium without serum and incubated all night in a medium

without serum in the presence of the same concentrations of HIV-PI. The supernatants of the cellular cultures were then collected and concentrated using Centricon-10 (Amicon, Bedford, MA). The protein concentration was determined by means of Bradford analysis (Bio-Rad, Hercules, CA) using the BSA as standard. Equal quantities (5 µg) of protein were then diluted in a buffer for zymography (5X) (0.4 M Tris-HCl, pH 6.8, 5% SDS, 20% glycerol and 0.03% bromphenol blue) and loaded on polyacrylamide gel with 9% of SDS containing 1 mg/ml of gelatine. After electrophoresis, the gels were incubated for 1 hour in 2.5% (v/v) of Triton X-100 to eliminate the SDS and subsequently with an enzymatic buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM CaCl2, 0.02% Brij-35) for the whole night at 37°C, as described previously (Kleiner et al., 1993). The gels were then stained with 2.5% Comassie blue G-250 and destained in 30% methanol and 10% acetic acid. The densitometry of the destained areas was then quantified using a densitometer GS-700 connected to a Macintosh Performa computer with Multi-Analyst software (Bio-Rad).

Fig. 7 Indinavir and saquinavir block the formation of KS lesions induced by primary KS cells in the nude mouse.

The nude mice were inoculated with KS cells ($3x10^6$) to induce the formation of angioproliferative KS lesions or with its re-suspension buffer (control) and treated with indinavir, saquinavir or saline solution according to the doses and procedures described in the key to figure 1. At the time of sacrifice, the sites of inoculation were examined to check the presence of macroscopic angioproliferative lesions as described in the key to figure 1. Panels a, b. 250X and 400X enlargements of the inoculation sites stained with haematoxylin and eosin of the mice injected with KS cells and treated with saline solution, respectively; panels c and d, 250X and 400X enlargements of the inoculation of the mice injected with KS cells and treated with indinavir, respectively; panels e and f, 250X and 400X enlargements of the inoculation of the mice injected with KS cells and treated with saquinavir, respectively. The experiments were carried out as described in the key to Table 2.

Fig. 8 Indinavir and saquinavir block the vascular permeability and oedema induced in the nude mouse by KS cells.

Nude mice were treated with indinavir, saquinavir or saline solution for 2 days with the same doses and procedures already described. On the third day they were inoculated with pyrilamine (80 µg in 100 µl of saline solution, 4 mg/kg, Sigma), to avoid the interference of the release of histamine due to inoculation, immediately afterwards with 100 µl of Evans blue (5 mg/ml in saline solution) endovenously and then subcutaneously with KS cells ($3x10^6$/mouse) cultivated in vitro in the presence of indinavir, saquinavir (1 µM) or of diluting buffer in 0.2 ml of Matrigel. As a control, each animal was inoculated contralaterally with the same volume of diluting buffer and Matrigel. After 18 hours the animals were sacrificed and the quantity of staining decanted in the inoculation site of the KS cells was measured at the level of the two largest perpendicular diameters by means of a gauge. The quantity of staining decanted was also assessed after taking skin from the inoculation site and quantified with the spectrophotometer after extraction with formamide for 24 hours at 56°C (Nakamura et al., 1992). The quantity of staining decanted was calculated after subtraction of the optical density measured on the control site. As shown in the figure, treatment with indinavir or saquinavir reduced the quantity of staining decanted by 39.8% ($p<0.05$) and 44.5% ($p<0.01$) respectively, in the case of quantification on the spectrophotometer, and by 43.5% and 47.5% respectively in the case of measurement with a gauge.

Fig. 9 Indinavir and saquinavir inhibit the invasive capacity of KS cells.

The figure shows the result of invasion trials carried out on KS cells cultivated in vitro for 5-6 days in the presence of indinavir or saquinavir or of the diluting buffer (control). Both drugs inhibited the capacity of the KS cells to invade the Matrigel membrane in a dose-dependent manner. In particular, both the HIV-PI inhibited invasion with respect to the levels observed in control KS cells ($p<0.05$).

The trial was performed as described in figure 4. In brief, the KS cells were cultivated for 5-6 days in the presence of indinavir or saquinavir (0.01 – 1 µM) or of the diluting buffer (saline solution). The cells were harvested and then plated in duplicate ($5x10^5$ in a culture medium containing 0.05% BSA) in the top compartment of the Boyden chamber, always in the presence of HIV-PI or buffer. bFGF (20 ng/ml) was placed in the bottom compartment as a chemoattractant.

After 6 hours the cells that invaded the matrigel membrane were stained and counted as described in the key to figure 4.

Fig. 10 Indinavir and saquinavir inhibit the production of cytokines by KS cells in vitro. The figure shows the quantities of IL-6 present in the supernatant of KS cells cultivated in the absence (control) or in the presence of indinavir (IND) or saquinavir (SAQ). The cells were plated in plates of 6 wells and cultivated for 5 days as described (Ensoli et al., 1990) in continuous presence or absence of indinavir or saquinavir at the concentrations of 0.1, 1 and 10 μM. On the fifth day, the culture medium was replaced with a medium without serum containing bovine blood albumin (0.05% weight/volume) in the presence of indinavir or saquinavir at the concentrations indicated. After 24 hours of incubation, the supernatants of the cultures were tested with an ELISA trial (R & D Systems, Minneapolis, MN, USA) to determine the quantity of IL-6 present in the medium. The quantity of IL-6 is expressed in picograms per ml of supernatant. The same tests were carried out for bFGF, VEGF, IL-1α and IL-1β by means of commercial ELISA. Both indinavir and saquinavir reduced the production of bFGF, VEGF, IL-1α, IL-1β and IL-6.

Example 1

The nude mice were treated with indinavir (1.4 mg/day), saquinavir (1 mg/day) or saline solution (negative control) by means of intragastric gavage once a day for 2 days (Kleiner et al., 1993). The mice were then inoculated with bFGF (1 μg) or with its diluting buffer in the presence of matrigel (Kleiner et al., 1993; Ensoli et al., 1994a; Samaniego et al., 1998; Barillari et al., 1999a). The treatment with indinavir, saquinavir or saline solution was carried out every day for 5 more days. The mice were then sacrificed and the inoculation areas examined both macroscopically and microscopically for the presence of KS-like angioproliferative lesions (Kleiner et al., 1993; Ensoli et al., 1994a; Samaniego et al., 1998; Barillari et al., 1999a). In agreement with the previous results (Ensoli et al., 1994a; Samaniego et al., 1998; Barillari et al., 1999a), the inoculation of 1 μg of bFGF promoted the development of angioproliferous lesions in 71% of the non treated mice (Table 1). On the contrary, treatment with indinavir or saquinavir reduced the percentage of mice that developed lesions from 28% to 25%, respectively ($p<0.05$) (Table 1): Figure 1A shows an example of these results. Treatment with indinavir

or saquinavir completely blocked the formation of the lesion or greatly reduced the dimensions of the lesions. The microscopic examination of the inoculation sites in the mice treated with indinavir or saquinavir showed a marked reduction of angiogenesis and of the infiltration of the cells in comparison with mice inoculated with bFGF and not treated with HIV-PI. In case of total regression, the histological picture of the tissues was similar or identical to the one observed in the mice injected with the buffer alone (Negative control) (Fig. 1B).

Example 2

To check which of the processes required for angiogenesis has been inhibited by indinavir or by saquinavir, experiments were carried out in proliferation, migration and invasion in response to bFGF on primary human endothelial cells cultivated in the presence or absence of scalar concentrations of indinavir or saquinavir which are of the same order and size as those present in the plasma of the individual treated (Deeks et al., 1997).

As shown in Fig. 2, the HIV-PI had no effect on the basal proliferation of endothelial cells or on that promoted by bFGF at any of the concentrations used (Fig. 2). Likewise, no effect was noted with indinavir or saquinavir on the survival of endothelial cells. In contrast, both the HIV-PI inhibited the migration (Fig. 3) and completely blocked the invasion of endothelial cells (Fig. 4) promoted by bFGF at all the concentrations studied.

Example 3

The migration and invasion of endothelial cells are mediated by the proteolytic action of active MMP-2 which degrades the basal vascular membrane which allows the endothelial cells to migrate, proliferate and form new vessels (Stetler-Stevenson, 1999). The MMP-2 is released by the endothelial cells as a proenzyme (latent MMP-2 72 kD) which is proteolytically activated on the cell surface in the 64/62 kD form by means of a complex mechanism which involves other proteases (Stetler-Stevenson, 1999). To check whether indinavir or saquinavir have any effect on the activity of MMP-2 in endothelial cells, experiments were carried out to measure gelatinolytic activity (Kleiner et al., 1993). Indinavir or saquinavir (Figure 5 and 6 respectively) have a minimum or even no effect on the synthesis of the latent MMP-2, while both block the conversion of the

latent MMP-2 in its active form (64/62 kD) in a dose-dependent manner (Fig. 5 and 6). These effects were observed after 24 hours of incubation of the cells with the same concentrations as the drugs present in the plasma of the patients treated (Deeks et al., 1997). Similar effects were also observed after 5 days of incubation in the cells in the presence of HIV-PI.

The results reported indicate that indinavir and saquinavir have direct anti-angiogenic effects,. The inhibition of angiogenesis and of the formation of KS-like lesions in mice induced by bFGF by the HIV-PI are due to the blocking of the migration and invasion of endothelial cells. These processes, in turn, are promoted by the action of MMP-2. Indinavir or saquinavir inhibit the conversion of the latent MMP-2 into its active form with mechanisms that have still to be determined. In fact, no homology was found between the sequence of the active site of the protease of HIV and MMP-2 or other MMP.

Even though other proteases, involved in the angiogenesis, growth and invasion of tumours, could be blocked by indinavir and by saquinavir, MMP-2 represents a key example to this effect. Moreover, it has been demonstrated that MMP-2 is induced by bFGF or other factors that induce angiogenesis (Ensoli et al., 1994a; Barillari et al., 1999b; Stetler-Stevenson, 1999), that both bFGF and MMP-2 are expressed in KS lesions (Ensoli et al., 1989; Ensoli et al., 1994a; Samaniego et al., 1998; Toschi et al., submitted), and that the inhibition of bFGF or of MMP-2 blocks angiogenesis and/or the formation of KS lesions (Ensoli et al., 1989; Ensoli et al., 1994a; Ensoli et al., 1994b; Stetler-Stevenson, 1999; Koivunen et al., 1999; Carmeliet and Jain, 2000) and blocks the tumour grouth in general (Koivunen et al., 1999; Carmeliet and Jain, 2000).

Example 4

These results have been confirmed by the effect of indinavir or saquinavir on the formation of KS-like lesions promoted by the inoculation of primary human KS cells in nude mice, a model in vivo widely amply used in preclinical studies of the efficiency of anti-KS therapies (Ensoli et al., 1994b; Koivunen et al., 1999; Sgadari et al., 2000). These angioproliferous lesions are transitory, of murine origin, and are developed in response to cytokines, such as bFGF and VEGF, IL-1, IL-6 and others, released by the KS cells (Ensoli et al., 1989; Ensoli et al., 1994a; Ensoli et

., 1994b; Fiorelli et al., 1995; Samaniego et al., 1995; Samaniego et al., 1997; Sgadari et al., 2000). The animals were treated with the same procedures and doses of indinavir or saquinavir used in the experiments illustrated previously.

As shown in table 2, the inoculation of KS cells induced the formation of KS-like lesions in 100% of the animals. Treatment with indinavir or saquinavir reduced the percentage of mice that developed a lesion to 50% and 17% respectively. On histological analysis, while the non treated lesions present an intense infiltration of cells, neoformed vessels, inflammatory infiltration and oedema, the treated lesions show a central zone of intense necrosis having cells with a pyknotic nucleus, a marked reduction of the infiltration of fusiform cells, of the formation of new vessels and of specific inflammatory infiltration (Figure 7).

Example 5

To demonstrate the effect of HIV-PI on vascular permeability and the oedema which is an important clinical component in KS, in angiogenesis, in tumours and in inflammatory diseases, experiments of vascular permeability were carried out in nude mice inoculated with KS cells which induce oedema because they produce cytokines with oedemigenic effects (VEGF, VEGF+bFGF, IL-1, IL-6, etc.). Nude mice were treated with indinavir, saquinavir or saline solution for 2 days according to the doses and procedures already described in example 1, inoculated endovenously with Evan blue and then injected with KS cells cultivated in vitro in the presence of indinavir or saquinavir (1 μM) or of diluting buffer. After 12 hours the animals were sacrificed, the stained area present on the site of inoculation of the KS cells was measured with a gauge and the quantity of staining decanted was measured in the spectrophotometer after extraction with formamide (Nakamura, Science 1992). As shown in figure 8, treatment with indinavir or saquinavir reduced the quantity of staining by 39.8% ($p<0.05$) and 44.5% ($p<0.01$) respectively, in the case of quantification on the spectrophotometer, and by 43.5% and 47.5% respectively in the case of measurement with a gauge (Figure 8).

Example 6

To study the mechanisms of the inhibiting effects of HIV-PI on the formation of KS lesions induced by KS cells, experiments of adhesion, proliferation, migration and invasion were carried out, cultivating the KS cells in the presence of indinavir or

saquinavir at concentrations between 0.01 μM and 1 μM for 5-7 days. As shown in table 3, indinavir or saquinavir do not inhibit the capability of the KS cells to adhere to a substrate of fibronectine. Likewise, treatment of the KS cells with indinavir or saquinavir for 7 days had no effect on cell proliferation measured by counting the vital cells stained with trypan blue (table 3).

To determine whether HIV-PI interfere with the capacity of KS cells to migrate and invade the basal membrane in response to angiogenic factors, KS cells treated for 5 days with indinavir or saquinavir (0.01 μM - 1 μM) were placed in the top compartment of Boyden chambers always in the presence of HIV-PI, while bFGF was placed in the bottom compartment as a chemoattractant. As shown in table 3, neither indinavir nor saquinavir had any effect on the migration of KS cells. In contrast, both drugs inhibited the capacity of the KS cells to invade in a dose-dependent manner. In particular, both HIV-PI inhibit the invasion of KS cells by 30-40% ($p<0.05$) (Table 3 and Figure 9).

Example 7

KS cells secrete cytokines with an inflammatory, oedemigenic, angiogenic and proliferative activity with autocrine and paracrine effects (Ensoli and Stürzl, 1998; Ensoli et al., in press). These factors mediate the formation of KS-like lesions in its various components (angiogenesis, cellular proliferation, inflammatory infiltration, oedema) and the vascular permeability and oedema induced by KS cells in nude mice. To determine the effects of indinavir and saquinavir on the production of cytokines, KS cells were cultivated in the presence or absence of scalar concentrations of indinavir or saquinavir. The quantity of bFGF, VEGF, IL-1 and IL-6 was dosed with immuno-enzymatic trials in the supernatants of the KS cells after 24 hours of culture in the absence of serum and in the continuous presence of the two HIV-PI (ELISA). Indinavir and saquinavir inhibited the production of bFGF, VEGF, IL-1α, IL-1β and IL-6 by KS cells. As an example of these effects, figure 10 shows the inhibition of IL-6, a typical inflammatory cytokine produced by KS cells and endothelial cells, but also by lymphocytes and monocytes of the blood and of the tissues and which also has angiogenic effects (Mateo et al., 1994; Cohen et al., 1996). Furthermore IL-6 plays a key role in the multicentric disease of Castleman and in the growth of lymphomas (Tosato et al.,

1993; Peterson and Frizzera, 1993; Asou et al., 1998; Ramsay et al., 1994), another type of tumour whose incidence is reduced in patients treated with HIV-PI (International Collaboration on HIV and Cancer 2000).

Example 8

The Tat of HIV increases the frequency and aggressiveness of KS in subjects infected with HIV-1 (Ensoli et al., 1994a). This is due to the induction by Tat of the adhesion, migration, invasion and proliferation of endothelial cells and of KS. In fact Tat synergistically increases the effects of bFGF on angiogenesis and on KS (Ensoli et al., 1994a; Barillari et al., 1999a and 1999b). However, Tat requires the presence of bFGF or of inflammatory cytokines which induce the production of bFGF to exert its action on KS, since bFGF increases the expression of the receptors for Tat on the cells and on the tissues (Barillari et al., 1992; Barillari et al., 1993; Albini et al., 1995; Fiorelli et al., 1995; Fiorelli et al., 1999; Barillari et al., 1999a; Barillari et al., 1999b).

Therefore, to check whether HIV-PI inhibit the effect of Tat and bFGF combining on angiogenesis and on KS, nude mice were inoculated with bFGF and Tat and treated with indinavir, saquinavir or with the buffer used for re-suspending them. As shown in table 4, both indinavir and saquinavir reduced the percentage of nude mice that developed KS lesions (50% and 20% respectively).

These results indicate that HIV-PI have specific inhibiting effects on angiogenesis and, consequently, on the growth of tumours, which need angiogenesis in order to grow and metastasise. HIV-PI also have direct inhibiting effects on the invasion of KS cells, on the growth of KS lesions, on vascular permeability and on the oedema induced in the mouse by KS cells. Moreover, HIV-PI inhibit the production of cytokines and other factors which mediate the formation of KS and the growth of other tumours and the inflammatory infiltration associated with them. HIV-PI also have anti-inflammatory effects as they reduce the production of cytokines such as IL-6, IL-1, and probably other cytokines involved in the inflammation and which are also present in human or mouse KS lesions. These same inflammatory cytokines are able to induce the production of angiogenic factors (bFGF, VEGF) and also have angiogenic effects in vivo (Barillari et al., 1992; Samaniego et al., 1995; Fiorelli et al., 1995; Samaniego et al., 1997; Samaniego et al., 1998; Fiorelli et al.,

1998; Fiorelli et al., 1999; Barillari et al., 1999a). In particular, IL-6 plays a key role in the multicentric disease of Castleman and in the growth of lymphomas (Tosato et al., 1993; Peterson and Frizzera, 1993; Ramsay et al., 1994; Asou et al., 1998).

HIV-PI link the active site of HIV protease, which belongs to the family of aspartil-proteases. It has recently been demonstrated that these drugs can inhibit an aspartil-protease fungina (Cassone et al., 1999). However, none of the known proteases which are involved in angiogenesis, in the growth and metastasis of tumours, in vascular permeability or in inflammation is an aspartil protease, nor have investigations into homology of sequence found any similarity between the active site of HIV protease and the proteases involved in these processes. The effects that we demonstrated on MMP, on cell motility, on angiogenesis, on KS, on vascular permeability and on the production of cytokines were therefore completely unforeseeable and could not have been expected.

In fact, although some studies suggested that HIV-PI have an effect on the cell metabolism (Deeks et al., 1997; André et al., 1998; Weichold et al., 1999; Ledru et al., 2000; Tovo, 2000), we have demonstrated that HIV-PI exert a direct anti-angiogenic, anti-tumoural, anti-oedemigenic and anti-inflammatory activity which is not connected with known aspartil-proteases nor with the effects of HIV-PI on the replication of HIV or of HHV-8. In fact, the models in vitro and in vivo of angiogenesis and of KS used in this study are without any infective agent.

The same results were obtained with both indinavir and saquinavir, which share a similar structure with the other HIV-PI, though with specific chemical substituents for each drug. So these data indicate that the activities of HIV-PI that we discovered for indinavir and saquinavir are a property common to all HIV-PI which agrees with the effects of the different HIV-PI observed in the individuals treated (Lebbé et al., 1998; Cattelan et al., 1999; International Collaboration on HIV and Cancer, 2000).

The above effects of HIV-PI are observed at the same drug concentrations present in the plasma of treated patients and which are fairly well tolerated by these individuals. Likewise, no toxic effects of indinavir or saquinavir were observed in the mice treated. Moreover, indinavir and saquinavir did not show any effects on the survival or on the growth of endothelial cells, but they selectively showed

effects on their migration and invasion, suggesting that the HIV-PI do not damage pre-existing vessels. Since cell motility and angiogenesis are essential not only for the development of KS, but also for the growth and metastasis of tumours (Carmeliet and Jain, 2000; Stetler-Stevenson, 1999), the results described so far indicate that HIV-PI are promising anti-angiogenic and anti-tumoural drugs. Moreover, the same results indicate that HIV-PI block the vascular permeability and inflammation induced by inflammatory cytokines and vascular permeability factors, and the production of cytokines with a key role in the multicentric disease of Castleman and in the growth of lymphomas (Tosato et al., 1993; Peterson and Frizzera, 1993; Ramsay et al., 1994; Asou et al., 1998). HIV-PI and those similar to or derivatives from them could therefore be exploited to block the angiogenesis, growth, invasion and metastasis of solid tumours and tumours of the blood, oedema and inflammation, and could thus be successfully used in the therapy of KS, of tumours, of angioproliferous pathologies, and of inflammatory and autoimmune diseases both in HIV-negative subjects and in subjects infected by HIV.

Table 1. Indinavir and saquinavir block angiogenesis and the formation of KS lesions KS lesions induced by bFGF in nude mice.

Treatment	Injection	N. of mice with macroscopic vascular lesions/N. of mice inoculated	%
Saline solution	Buffer	0/18	0
Saline solution	bFGF	20/28	71
Indinavir	bFGF	8/28	28
Saquinavir	bFGF	7/28	25

The nude mice were inoculated with bFGF (1 μg) to induce the formation of KS-like angioproliferative lesions or with its re-suspension buffer (control) and treated with indinavir, saquinavir or saline solution. At the time of sacrifice, the inoculation sites were examined to check for the presence of macroscopic angioproliferative lesions. Here are listed the number (N.) of mice that developed lesions with

respect to the number (N.) of mice inoculated, and the percentage (%) of mice that developed lesions. The reduction of the number of KS-like lesions in the treated animals is statistically significant (standard test for the proportions, $p<0.05$).

The same formulas of indinavir (Merck-Sharpe & Dhome Ltd., Haarlem, NL) or saquinavir (Roche, Hertfordshire, GB) used in patients infected with HIV were dissolved in a saline solution and administered to nude mice (Balb/c nu/nu females, Charles River, Calco, Italy, 5-6 weeks old) by means of intragastric gavage. To test their toxicity, indinavir and saquinavir were administered once a day for a total of 8 days in doses of 35, 70 or 17.5 mg/Kg/day or of 18, 36 or 9 mg/Kg/day respectively, in a volume of 0.4 ml. These doses correspond, respectively, to the whole dose, double or half the dose of HIV-PI used daily in patients infected with HIV (Deeks et al., 1997). No organ toxicity or systemic toxicity was observed for any of these doses. The mice were treated with 70 mg/Kg/day of indinavir (corresponding to 1.4 mg/day) or with 36 mg/Kg/day of saquinavir (corresponding to 1 mg/day) once a day for a total of 7 days, starting from two days prior to the inoculation of bFGF. The control animals were treated with the same volume of saline solution. On the third day the mice were inoculated subcutaneously at the level of the lower dorsal quadrant with 1 μg of recombining bFGF (Roche, Mannheim, Germany) diluted in 0.2 ml of phosphate buffer (PBS)-0.1% bovine blood albumin (BSA) or with its re-suspending buffer, mixed with 0.2 ml of Matrigel (Collaborative Biomedical Products, Bedford, MA) prior to inoculation, as described previously (Ensoli et al., 1994a). Four days afterwards the mice were sacrificed, the inoculation zones were examined to check the presence of macroscopic KS-like angioproliferative lesions, and sections of tissue histologically examined after staining with haematoxylin/eosin.

Table 2. Indinavir and saquinavir block the formation of angioproliferous KS lesions promoted by inoculation of primary KS cells in nude mice.

Inoculant	Therapy	N. of mice with macroscopic vascular lesions/N. of mice injected %
KS cells	Saline	4/4 (100)

Indinavir	3/6 (50)
Saquinavir	1/6

The nude mice were inoculated with KS cells ($3x10^6$) to induce the formation of KS-like angioproliferative lesions or with its re-suspension buffer (control) and treated with indinavir, saquinavir or saline solution according to the doses and procedures described in the key to figure 1. At the time of sacrifice, the sites of inoculation were examined to check the presence of macroscopic angioproliferous lesions as described in the key to figure 1. Here are listed the number (N.) of mice that developed lesions with respect to the number (N.) of mice inoculated, and the percentage (%) of mice that developed lesions. The histological picture of the inoculation sites is shown in figure 7.

Table 3. Effects of indinavir and saquinavir on KS cells in vitro.

	Indinavir (μM)*			Saquinavir (μM)*		
KS Cells	0.01	0.1	1	0.01	0.1	1
Adhesion	ND	ND	1.05	ND	ND	1
Proliferation	1	1.08	1.11	1	1.15	1.25
Migration	ND	ND	1.03	ND	ND	1.21
Invasion	0.98	0.74**	0.71**	1	0.79**	0.62**

* Increase expresses in folds towards the control (1 fold)

**$p<0.05$

ND, not determined

The experiments in adhesion, proliferation, migration and invasion were carried out cultivating KS cells in the presence of indinavir or saquinavir at concentrations between 0.01 and 1 μM for 5-7 days. Iindinavir or saquinavir do not inhibit the capacity of the KS cells to adhere to substrate of fibronectine. Likewise, treatment of the KS cells with indinavir or saquinavir for 7 days had no effect on cell proliferation measured by counting the vital cells stained with trypan blue.

Neither indinavir nor saquinavir had any effect on the migration of KS cells. In contrast, both the drugs inhibited the capacity of the KS cells to invade the membrane of matrigel in a dose-dependent manner ($p<0.05$).

For the migration and invasion experiments, KS cells treated for 5 days with indinavir or saquinavir (0.01 µM - 1 µM) were placed in the top compartment of Boyden chambers always in the presence of HIV-PI, while bFGF was placed in the bottom compartment as a chemoattractant.

Table 4. Indinavir and saquinavir block the formation of angioproliferative KS lesions promoted by the inoculation of associated bFGF and HIV-Tat in the nude mouse

Inoculant	Therapy	N. of macroscopic vascular lesions/N. of mice injected (%)
Buffer	Saline solution	0/18 (0%)
bFGF+ Tat	Saline solution	7/10 (70%)
bFGF+ Tat	Indinavir	5/10 (50%)
bFGF+ Tat	Saquinavir	2/10 (20%)

The nude mice were inoculated with bFGF (1 µg) and Tat (10 µg) in association to induce the formation of KS-like angioproliferous lesions or with its re-suspending buffer (control) and treated with indinavir, saquinavir or saline solution. At the time of sacrifice, the inoculation sites were examined to check for the presence of macroscopic angioproliferative lesions. Here are listed the number (N.) of mice that developed lesions with respect to the number (N.) of mice inoculated, and the percentage (%) of mice that developed lesions. The mice were treated with indinavir and saquinavir according to the procedures and doses described in table 1 for a total of 7 days, starting from two days prior to the inoculation of bFGF and Tat. The control animals were treated with the same volume of saline solution. On the third day the mice were inoculated subcutaneously at the level of the lower dorsal quadrant with 1 µg of recombining bFGF (Roche, Mannheim, Germany) and 10 µg of HIV-1 Tat diluted in 0.2 ml of PBS-0.1% BSA or with its re-suspending buffer, mixed with 0.2 ml of Matrigel prior to inoculation, as described previously

(8). Four days afterwards the mice were sacrificed, the inoculation zones were examined to check the presence of macroscopic KS-like angioproliferative lesions, and sections of tissue histologically examined after staining with haematoxylin/eosin.

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CLAIMS

1. Use of the inhibitors of the protease of the virus HIV, HIV-PI, for the preparation of drugs for the therapy of angiogenesis.
2. Use of the inhibitors of the protease of the virus HIV, HIV-PI, for the preparation of drugs for the therapy of non neoblastic angioproliferative diseases.
3. Use according to claim 2 in which the non neoblastic angioproliferative diseases are selected among: diabetic retinopathy, retrolental fibroplasia, trachoma, vascular glaucoma, psoriasis, immune and non immune inflammation, atherosclerosis, keloids.
4. Use of the inhibitors of the protease of the virus HIV, HIV-PI, for the preparation of drugs for the therapy of tumours.
5. Use according to claim 4 in which the tumour is selected among: benign and malignant tumours of the soft tissues, the cartilages, the bones and the blood.
6. Use of the inhibitors of the protease of the virus HIV, HIV-PI, for the preparation of drugs for the therapy of Kaposi's sarcoma (KS).
7. Use of the inhibitors of the protease of the virus HIV, HIV-PI, for the preparation of drugs for blocking the activity of the bFGF, with a therapeutic anti-angiogenic, anti-KS and anti-tumoural effect.
8. Use of the inhibitors of the protease of the virus HIV, HIV-PI, for the preparation of drugs for blocking the activity of the VEGF, with a therapeutic anti-angiogenic, anti-KS, anti-tumoural and anti-oedemigenic effect.
9. Use of the inhibitors of the protease of the virus HIV, HIV-PI, for the preparation of drugs for blocking the activity of associated bFGF and VEGF, with a therapeutic anti-angiogenic, anti-KS, anti-tumoural and anti-oedemigenic effect.
10. Use of the inhibitors of the protease of the virus HIV, HIV-PI, for the preparation of drugs for blocking the activity of Tat alone or in the association with bFGF, with a therapeutic anti-angiogenic, anti-KS, anti-tumoural, anti-oedemigenic and anti-inflammatory effect in subjects with HIV infection.

11. Use of the inhibitors of the protease of the virus HIV, HIV-PI, for the preparation of drugs for blocking the migration of endothelial cells, with a therapeutic anti-angiogenic, anti-KS and anti-tumoural effect.

12. Use of the inhibitors of the protease of the virus HIV, HIV-PI, for the preparation of drugs for blocking the migration of tumoural cells with a therapeutic anti-KS and anti-tumoural effect.

13. Use of the inhibitors of the protease of the virus HIV, HIV-PI, for the preparation of drugs for blocking the invasion of endothelial cells, with a therapeutic anti-angiogenic, anti-KS and anti-tumoural effect.

14. Use of the inhibitors of the protease of the virus HIV, HIV-PI, for the preparation of drugs for blocking the invasion of tumoural cells with a therapeutic anti-KS and anti-tumoural effect.

15. Use of the inhibitors of the protease of the virus HIV, HIV-PI, for the preparation of drugs for blocking MMP-2 and the other proteases involved in angiogenesis.

16. Use of the inhibitors of the protease of the virus HIV, HIV-PI, for the preparation of drugs for blocking the proteases involved n the growth and metastasis of tumours.

17. Use of the inhibitors of the protease of the virus HIV, HIV-PI, for the preparation of drugs for blocking vascular permeability and oedema associated with angiogenesis.

18. Use of the inhibitors of the protease of the virus HIV, HIV-PI, for the preparation of drugs for blocking vascular permeability and oedema associated with inflammation.

19. Use of the inhibitors of the protease of the virus HIV, HIV-PI, for the preparation of drugs for blocking vascular permeability and oedema associated with tumours.

20. Use of the inhibitors of the protease of the virus HIV, HIV-PI, for the preparation of drugs for blocking vascular permeability and oedema associated with KS.

21. Use of the inhibitors of the protease of the virus HIV, HIV-PI, for the preparation of drugs for the therapy of autoimmune diseases.

22. Use according to claim 21 in which the autoimmune disease is selected among: rheumatoid arthritis, systemic lupus erythematosus, thyroiditis, Goodpasture's syndrome, systemic vasculitis, sclerodermia, Sjögren's syndrome, primitive biliary cirrhosis, ulcerous rectocolitis and Crohn's disease, psoriasis.

23. Use of the inhibitors of the protease of the virus HIV, HIV-PI, for the preparation of drugs for the therapy of inflammatory diseases.

24. Use according to claim 23 in which the inflammatory disease is selected among: allergies and chronic inflammations associated with viral infective, bacterial or parasitic agents, including the multicentric disease of Castleman.

25. Use of the inhibitors of the protease of the virus HIV, HIV-PI, for the preparation of drugs for blocking the production of inflammatory cytokines, with a therapeutic anti-inflammatory, anti-oedemigenic, anti- angiogenic, anti-KS and anti-tumoural effect.

26. Use according to claims 1-25 in which the inhibitor of the protease of the virus HIV, HIV-PI, is selected among: indinavir, saquinavir, ritonavir, nelfinavir, amprenavir, lopinavir, lopinavir and those similar to or derivatives from them, alone or in combination among them and/or in combination with anti-inflammatory, anti-angiogenic or anti-tumoural drugs.

27. Use according to claims 1-26 in subjects infected by HIV.

28. Use according to claims 1-27 to be administered according to a procedure selected among; oral, intravenous, intramuscular, subcutaneous, intradermal, intraperitoneal, intrathecal, intrapleural, intrauterine, transmucosal, rectal, vaginal, intralesional or percutaneous administration.

29. Use according to claims 1-28 in which the dose is selected among the following: for indinavir: 1200 mg/day, 2400 mg/day and 4800 mg/day; and for saquinavir: 1800 mg/day, 3600 mg/day, 7200 mg/day.

Rome, 18.04.2001

f. ISTITUTO SUPERIORE DI SANITA'

The Representative

(signature)

Maria Vittoria Primiceri